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14. ABSTRACT BRCA1 C-terminal (BRCT) domains are novel phosphopeptide binding modules. Cancer-associated missense and deletion mutations have been found in the BRCT repeat regions of BRCA1, suggesting an essential role of BRCT domains in regulating BRCA1 activity. In addition, BRCT domains are found in many proteins that regulate DNA damage repair, cell cycle, and genome stability, implying a more global role of BRCT domains in genome stability surveillance. These results suggest that the BRCT domain acts as a sensor to protein phosphorylation in response to DNA damage, recruits phosphorylated cellular targets, and mediates signaling complex formation. However, the identities of the in vivo BRCT domain targets are largely unknown. In order to understand the role of phosphorylation in protein-protein interactions, we developed several approaches utilizing peptide libraries and peptide arrays. We propose to use these methods to systematically identify phosphoproteins that can interact with BRCT domains. In addition to potential new regulators of genome stability, the approaches can identify phosphorylated sequences on proteins that are important for DNA damage responses and cell cycle. Such information should prove valuable, especially for the development of new screening strategies, drug targets, and treatment for breast cancer.					
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Introduction

Understanding the molecular and cellular mechanisms that trigger breast cancer is essential to the prevention and treatment of this disease. The BRCA1 C-terminal (BRCT) domain was first identified in BRCA1 (1, 2). Cancer associated missense and deletion mutations have been found in the BRCT repeat regions of BRCA1, suggesting an important role of BRCT domains in regulating BRCA1 activity (6, 9). In addition, the BRCT domain is found in many proteins that regulate DNA damage repair, cell cycle, and genome stability, implying a more global role of BRCT domains in genome stability surveillance (1, 2). Consistent with this notion, the BRCT domain has been shown to mediate protein-protein interactions. For example, BRCT domains of BRCA1 associate with helicase BACH1 and CtBP interacting protein CTIP (3, 13). Recently, our lab and others have discovered that BRCT domains are novel phosphopeptide binding modules (8, 10, 12). BRCA1 BRCT domains associate with residue Ser990 on BACH1 in a phosphorylation-dependent manner. Furthermore, we found that several other BRCT domains including those from MDC1 and tumor suppressor BARD1 can bind specific phosphorylated peptides (4, 7, 11). These findings suggest that the BRCT domain recruits phosphorylated cellular targets and mediates signaling complex formation. However, the identities of the *in vivo* BRCT domain targets are largely unknown. In this application, we propose to systematically identify phosphoproteins that can interact with BRCT domains. Through these efforts, we may uncover potential new regulators of genome stability; more importantly, the approach can identify phosphorylated sequences on proteins that are important for DNA damage responses and cell cycle. Such information will help us to understand the mechanism of how protein phosphorylation modulates DNA damage responses and cell cycle in breast epithelial cells. In addition, it should prove invaluable for the development of new screening strategies and treatment for breast cancer.

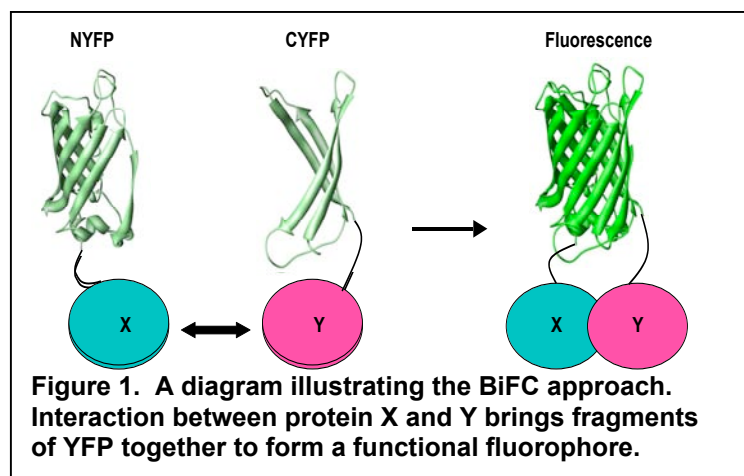
Body

A. For Task 1, we have proposed to identify phosphorylated peptide sequences that specifically bind BRCA1 and BARD1 BRCT domains. We planned to utilize Oriented Peptide Array Libraries (OPAL) to examine the specificity of BRCA1 and BARD1, and peptide chips to screen for BRCA1 and BARD1 BRCT domain binding targets.

1.1 In our last report, we proposed to optimize OPAL synthesis in order to minimize background binding while retaining high specificity. This has proven extremely difficult. To date, we have experimented with several different synthesis surfaces as well as reaction protocols. And we have synthesized larger arrays as an alternative to circumvent some of the technical problems. But each method and synthesis surface carries their own limitations that significantly hinder the progress of OPAL-mediated target screening.

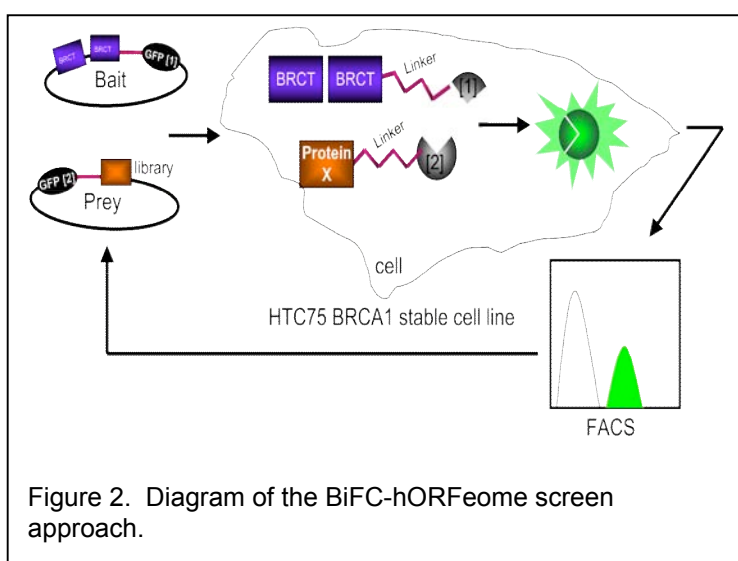
In order to identify binding targets for BRCA1, BARD1, and BRCT domains, we worked on establishing a novel *in vivo* binding screening strategy that takes advantage of Bi-molecular Fluorescence Complementation (BiFC), while continuing to optimize the OPAL arrays.

(1) BiFC-hORFeome design to screen for BRCA1 and BRCT targets



Bimolecular fluorescence complementation (BiFC) was originally developed to visualize protein-protein interactions in live cells (5). Two separate proteins are respectively fused to the N- or C-terminal fragment of a fluorescence protein (e.g., YFP). When the two proteins interact, the YFP fragments will be brought to close proximity to form a functional fluorescent complex (Figure 1).

BiFC offers several advantages for establishing protein-protein interactions. For example, it enables visualization of interactions in live cells, allows for the examination of the subcellular locations of specific protein-protein interactions, and it is highly amenable to investigating inducible interactions.

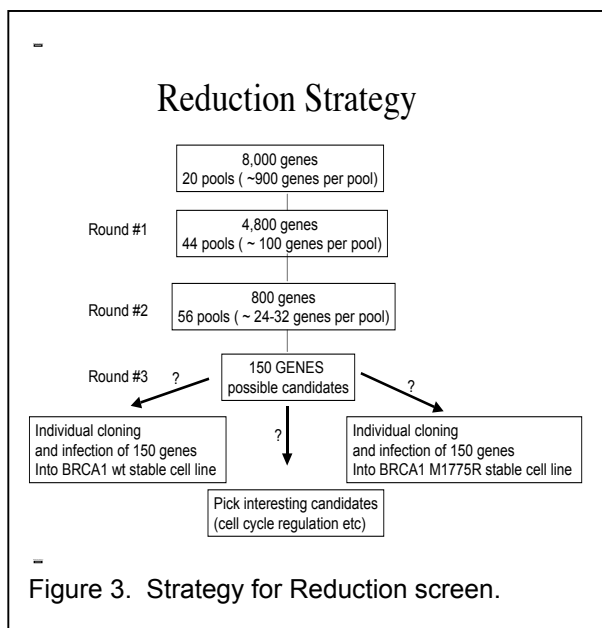


To develop the BiFC technology for screening for BRCT-BRCA1 interacting proteins in mammalian cells, we constructed expression vectors using the Gateway® cloning system. These vectors were designed to encode either the N- or C-terminal half of YFP (YFPn and YFPc respectively). For the bait, we engineered BRCA1 BRCT domain sequences tagged by YFPn and established stable cell lines expressing YFPn-BRCT.

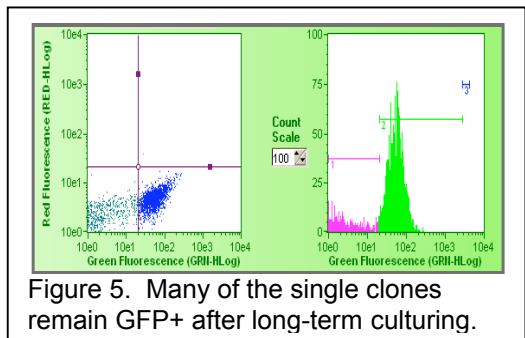
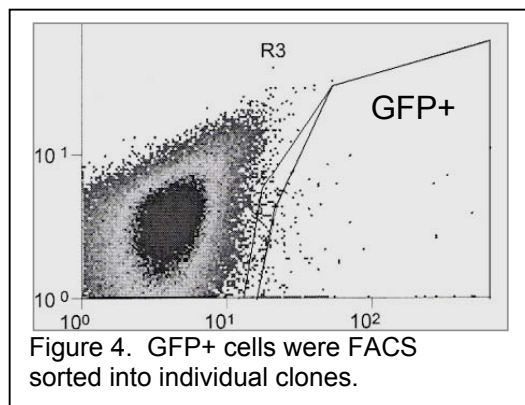
Next, we generated YFPc-tagged cDNA libraries from the Human Open Reading Frame Collection (hORFeome, Openbiosystems). The hORFeome contains ~8,000 human individual open reading frames and was used as prey in our studies (Figure 2).

(2) Pilot BiFC-hORFeome screens for BRCT domain

To screen for BRCA1-BRCT interacting proteins using BiFC, we undertook a reduction approach. We divided the ~8,000 hORFeome into pools. Each pool was then



used to generate high-titer retroviruses for subsequent infection of the YFPn-tagged BRCT-BRCA1 expressing stable cell line.



chromosome maintenance, and cell cycle control. An additional screen also yielded ~130 clones. Preliminary analysis of the clones revealed factors previously unsuspected in DNA damage pathways. These include PTK9L, a protein tyrosine kinase involved in actin depolymerization, and NOL5A, a nucleolar protein that contains putative snoRNA binding domains (Table 1).

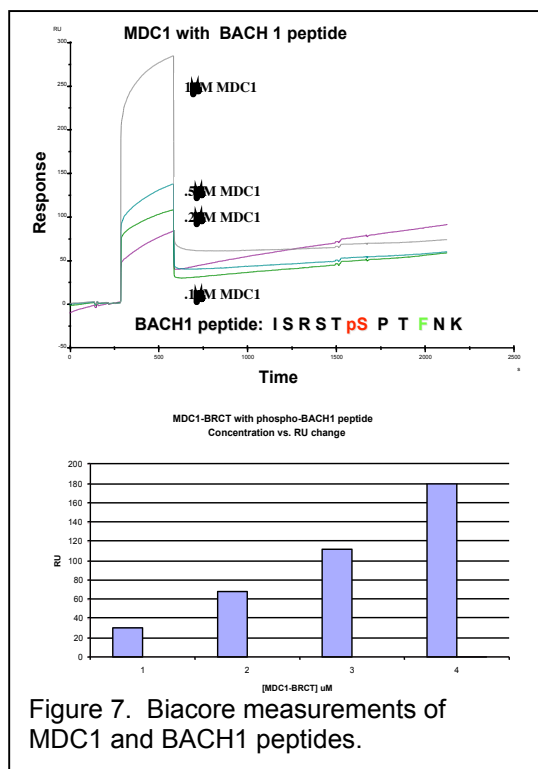
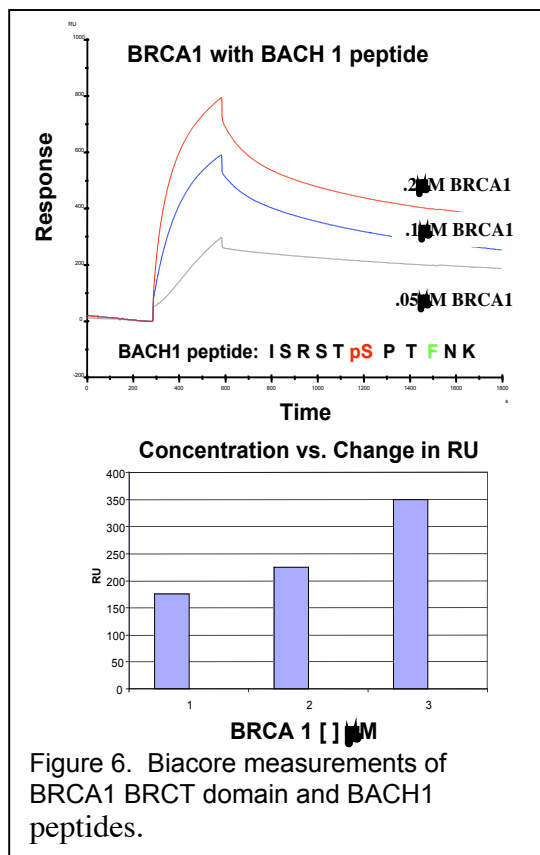
It has been demonstrated that the BRCT motif is important for BRCA1 nuclear localization (nuclear foci) during S phase and its recruitment to double-stranded breaks (DSB) after irradiation (IR). These foci likely represent sites of DNA damage, because of the co-localization with the DNA repair protein hRad51 and ATM-phosphorylated substrates such as γ -H2AX, a known marker of radiation-Induced DSBs. Therefore, how the interactions between BRCA1-BRCT and its target are affected by treatments such as

Interaction between YFPn-BRCA1-BRCT and YFPc-tagged prey proteins would bring YFPn and YFPc to close proximity and allow for the assembly of a functional fluorescent complex. The cells can thus be analyzed by fluorescence-activated cell sorting (FACS) to examine the intensity of the fluorescence and by microscopy for the localization.

Indeed, we obtained many cell clones that exhibited either strong or weak fluorescence (Figure 4), indicating that these cells may express potential BRCA1 interacting proteins. These sorted positive cells were further propagated, sorted, and subdivided again. Many remained GFP+ after long-term culturing (Figure 5). After three rounds, we were able to narrow down the number of BRCT-BRCA1 candidate binders from ~8,000 genes to ~150 genes. These ~150 candidates are involved in diverse cellular functions including ubiquitination,

	Protein	Putative recog. site	Description
HSA9761	Dimethyladenosine transferase	YQISSPEVF KTLAAAFK II TSTGESD	Dimethyladenosine transferase (rRNA methylation) [Translation, ribosomal structure and biogenesis]
PKIB	protein kinase (cAMP-dependent, catalytic)	?	interact with the catalytic subunit of cAMP-dependent protein kinase and act as a competitive inhibitor
CAMKV	hypothetical protein MGC8407	?	Phosphotransferases of the serine or threonine-specific kinase subfamily.
MAMDC2	MAM domain containing 2	VEASCNFEQ PAGSCAFEE QNSSKKFK	MAM domain. An extracellular domain found in many receptors.
ING5/4	inhibitor of growth family, member 5	KLVRTSPEYG GMPSVTFGSV KQIESSDYDS	a tumor suppressor protein that can interact with TP53, inhibit cell growth, and induce apoptosis
NACAP1	nascent-polypeptide-associated complex alpha polypeptide	SPASDTYIV	
PTK9L	PTK9L protein tyrosine kinase 9-like (A6-related protein)	DPLESVVEFY	Actin depolymerisation factor/cofilin-like domains; these proteins enhance the turnover rate of actin
LOC124220	Similar common salivary protein	GIKSIGFEW GQISSAYPS	
DOK3		ALYSWPYHF NDLASGLYAS SPTTSPYIYN	adapter molecule involved in the negative regulation of immunoreceptor signaling
NOL5A	nucleolar protein 5A	MEDPSISFSK	Putative snoRNA binding domain. This family consists of various Pre RNA processing ribonucleoproteins.

Table 1. A select list of genes identified from BiFC screen.



IR may be examined. Such analysis would offer clues to the importance of these interactions to DNA damage responses. We will also individually confirm these genes for their interaction with BRCA1-BRCT.

1.2 During the past year, we have also carried out biochemical studies of BRCT domains and their targets.

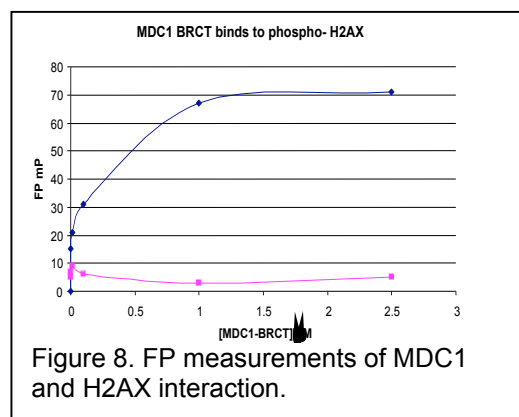
Surface Plasmon Resonance (SPR) or Fluorescence Polarization (FP) were used for such studies. When comparing interaction between phosphorylated and unphosphorylated peptides, an aliquot of the phosphorylated peptides was dephosphorylated with alkaline phosphatase for controls.

a. SPR is highly sensitive and can measure very low affinities and analyze kinetics data. SPR requires the immobilization of peptides or proteins onto a sensor chip. After trying multiple procedures, we were able to determine the concentration of the peptide as well as the immobilization method that better suits our studies.

First, the biotin-BACH1 peptide was immobilized onto a streptavidin-coated sensor chip. Next, the chip was incubated with different concentrations of BRCA1 GST-BRCT fusion proteins to determine the *on* and *off* rates of the interaction. Based on these preliminary results, wildtype BRCA BRCT clearly discriminates between the phosphorylated vs. the unphosphorylated peptide (Figure 6). To further confirm the specificity of this interaction, a control MDC1 BRCT domain was used, because it interacts much weaker with the BACH1 sequence (Figure 7). Our results showed the kinetics of BRCA1 BRCT interaction with the phosphorylated BACH1 peptide. These results have validated our system and conditions, and contributed to our understanding of the specificity of the BRCT domains.

b. In general, FP is of very low background, because the interaction occurs in solution without the need to immobilize the protein or peptide. For the FP studies, we examined the BRCT domain of MDC1 histone gamma H2AX (γ -H2AX)(Fig. 8). Briefly, phosphorylated and

unphosphorylated forms of FITC-labeled γ -H2AX peptides were incubated with increasing concentrations of MDC1 BRCT domains. The concentration of MDC1-BRCT vs. measurements of fluorescence polarization was plotted and K_d calculated. These studies have been carried out with wild type BRCA1 BRCT, and conditions have been optimized for further studies with the mutant form of the BRCA1 BRCT domain.



B. For Task 2, we proposed to characterize breast cancer genes and BRCT binding sites identified in Task 1, by RNAi and by determining the role of BRCT-phosphopeptide interaction in DNA damage.

While we are optimizing screening conditions and generating assay reagents, we have obtained more results with BARD1, the tumor suppressor protein that interacts with BRCA1 and contains a BRCT domain that can bind specific phosphorylated peptides as well. As mentioned in

the last report, three retroviral RNAi vectors for BARD1 have been made. We also have obtained two different antibodies for human BARD1. Our pilot studies indicate that these RNAi constructs could significantly knock down BARD1 (>70%). Furthermore, in cells where expression of BARD1 was inhibited, the cells appeared to accumulate in G2/M. These findings are being further investigated and confirmed. In addition, we are currently carrying out similar BiFC-hORFeome screens for BARD1.

Key Research Accomplishments

- We have carried out BiFC in vivo interaction studies and obtained a number of novel interaction partners for BRCA1-BRCT domains.
- We have demonstrated that BiFC interaction screens are not only suitable for gene identification, but also offer important clues to the localization of the interaction in live cells.
- We have obtained kinetic data regarding BRCA1 BRCT domain and phosphorylated BACH1 peptide interaction.
- We have shown by both Biacore and FP the interaction between MDC1 BRCT domains and its potential targets.
- We have successfully generated BARD1 knockdown cells and carried out preliminary analysis of these cells.

Reportable Outcomes

Meeting abstract/presentation

BRCT domains, targets and breast cancer. (2006) Maria Rodriguez, Zhou Songyang. SCBA Annual Research Conference. Houston, TX

Conclusions

In summary, we have conducted pilot screens of BRCT domain interacting sequences using BiFC. We have obtained a number of potential targets for further confirmation and examination. The information obtained from our studies should prove especially useful for the development of new and effective screening strategies, drug targets, and treatment for breast cancer.

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